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Extraction and Purification of Curcain, a Protease from the Latex of *Jatropha curcas* Linn

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Abstract—A proteolytic enzyme, curcain, has been extracted from the latex of *Jatropha curcas* Linn. The enzyme was purified by chromatography on carboxymethyl cellulose and gel filtration on Sephadex G-200. The homogeneity of protein associated with curcain was established by non-denatured polyacrylamide gel electrophoresis using a discontinuous buffer system. The molecular weight of curcain was estimated by Sephadex G-100 gel filtration using a calibration curve of standard proteins to be around 22 000 daltons.

Jatropha curcas Linn (Family Euphorbiaceae) is a perennial hedge plant grown in India and other tropical countries (Chopra et al 1958; Hooker 1973). All parts of the plant exude a sticky, opalescent, acrid and astringent latex (Sastri 1959) that dries to a bright reddish brown, brittle substance, resembling shellac. Almost every part of the plant is credited with some medicinal properties (Chopra et al 1956, 1958; Sastri 1959). As powder of the leaves is applied to wounds and refractory ulcers, and the latex of the plant is applied to decayed teeth and to wounds, and is used as a styptic in ordinary cuts and bruises, the presence of proteolytic activity might be anticipated.

There have been a number of investigations on the chemical and biological active constituents of *Jatropha curcas* Linn. Analysis of seeds gave the following values (Sastri 1959) (%): moisture 6·62; protein 18·2; fat 38·0; carbohydrate 17·98; fibre 15·5 and ash 4·5%. Mitra et al (1970) isolated the β-D-glucoside of β-sitosterol, dulcitol and sucrose from the stem bark. Felke (1914) isolated and identified the toxic principle curcin, a toxalbumin from the seeds of the plant. Khafagy et al (1977) isolated stigmasterol and β-sitosterol from the light petroleum extract of the leaves and a dimer of a triterpene alcohol $C_{63}H_{117}O_9$ from the ether extract of the leaves. Adolf et al (1984) isolated a polyunsaturated diterpene ester, 12-deoxy-10-hydroxyphorbol from the seed oil of *J. curcas* which is irritant and purgative. Several flavonoids and flavonoidal glycosides had also been isolated from the leaves (Subramanian et al 1971).

Some members of the Euphorbiaceae have proteolytic enzymes in their latex, but no proteolytic enzyme had been found in *J. curcas* Linn, until preliminary investigations by the authors confirmed the presence of such enzyme in the latex of the plant. Some of the physicochemical properties of the partially purified protease were reported by Nath & Dutta (1988). The pH for optimum enzyme activity was found to be 5·0. The maximum activity of the enzyme was registered at 45°C and the isoelectric pH of the enzyme was 5·5–6·0. The aim of this study was to purify the protease so that it could be used as a substitute for other available proteolytic enzymes.

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Materials and Methods

Materials

Latex of *Jatropha curcas* Linn (150 mL) was freshly collected by nipping the tender shoots. Carboxymethyl cellulose (CM-cellulose) (capacity 0·72 meq g^{-1}), Sephadex G-200 (40-120 μm) and Sephadex G-100 (40–120 μm) were from Pharmacia Fine Chemicals, Sweden. Blue dextran 2000 (mol. wt 2×10^6), bovine serum albumin (fraction V, mol. wt 66 000), ovalbumin (chicken egg, mol. wt 45 000) and cytochrome C (horse-heart, mol. wt 12 400) were used as standard proteins and were purchased from Sigma Chemical Co., USA. Acrylamide, *N*,*N*-methylenebisacrylamide (BIS), ammonium persulphate, *N*, *N*, *N'*, *N'*-tetramethylethylene diamine (TEMED) and Coomassie Brilliant Blue (R 250) were of electrophoretic grade and were obtained from Sigma Chemical Co., USA. Azoalbumin substrate was prepared in our laboratory according to the method of Tomarelli et al (1949). Dialysis tubing was purchased from the V.P. Chest Institute, CSIR Centre for Biochemicals, New Delhi. The buffer solutions were prepared according to Gomori (1955). All other chemicals were either obtained from Qualigens Fine Chemicals or E. Merck, India Ltd and were standard laboratory reagents of analytical grade.

Protease assay

The assay of proteolytic activity was performed using azoalbumin as substrate at 45°C, pH 5·0 by measuring the colour intensity of digested product at 440 nm (Tomarelli et al 1949). The unit of activity was defined (Kunitz 1947) as the amount of protease which caused an increase of one unit of absorbancy at 440 nm per min of digestion. Specific activity was expressed as the number of units of activity per mg of protein.

Protein estimation

Protein was measured according to the method of Warburg & Christian (1942) spectrophotometrically at 280 nm assuming that a reading of one unit of absorbancy corresponds to a protein concentration of 1 mg mL^{-1}.

Enzyme extraction

To 150 mL of latex, 2 vol of distilled water was added and the mixture was kept overnight in the deep-freeze (−7°C). The

floating gum was separated out, and the latex solution centrifuged at 5000 rev min^{-1} for 30 min at 5°C. The supernatant crude extract (400 mL) was used as the starting material (Fraction 1). The crude extract was divided into three parts for extraction of enzyme by three different methods: acetone precipitation, mixed-solvent precipitation and ammonium sulphate fractionation.

Chilled acetone (300 mL) was added to 100 mL of cooled crude extract. The precipitate formed was separated by centrifugation, washed with acetone and dissolved in the minimal amount of water and reprecipitated by adding chilled acetone. The precipitate collected after centrifugation was dissolved in 50 mL of 0·2 M acetate buffer, pH 5·0 (Fraction 2).

To 100 mL of cooled crude extract, 200 mL of chilled absolute alcohol was added. The precipitate was separated by centrifugation, dispersed in the minimum amount of water and reprecipitated by addition of chilled acetone (1:3). The enzyme collected from the centrifuge tube was washed with acetone and dissolved in 50 mL of 0·2 M acetate buffer, pH 5·0 (Fraction 3).

Ammonium sulphate (28·8 g) was added slowly to 200 mL of cooled crude extract to make the solution 25% saturation with constant stirring over a period of 10 min. The mixture was kept in a refrigerator for 30 min and then cold-centrifuged (5°C) at 5000 rev min^{-1} for 30 min. The precipitate collected was dissolved in 50 mL of 0·2 M acetate buffer, pH 5·0 (Fraction 4).

The supernatant obtained from Fraction 4 was brought to 60% saturation with solid ammonium sulphate as before and the precipitate obtained was dissolved in 50 mL of buffer (Fraction 5).

The supernatant obtained from Fraction 5 was treated with the calculated amount of ammonium sulphate to make 80% saturation in the same way as described above and the precipitate collected by cold-centrifugation was dissolved in buffer (Fraction 6).

Fraction 4 was dialysed for 24 h against 5 L of acetate buffer (0·2 M, pH 5·0) with several changes (Fraction 7).

Carboxymethyl cellulose chromatography of Fraction 7

A column (1·5 × 15 cm) of CM-cellulose was prepared by the procedure of Peterson & Sober (1962) and equilibrated with 0·1 M acetate buffer, pH 5·0. Four mL of Fraction 7 was loaded on to the column and eluted stepwise with 0·05, 0·1, 0·2, 0·3, 0·5 and 0·7 M NaCl in the same buffer. Effluents were collected in 5·0 mL fractions at a flow rate of 15 mL h^{-1}. The eluted fractions showing peak activity were pooled (50 mL) and dialysed against deionized water (Fraction 8).

Gel filtration of Fraction 8 with Sephadex G-200

The method of Whitaker (1963) was used for gel filtration of Fraction 8. A Sephadex G-200 column (2·0 × 45 cm) was equilibrated with 0·1 M acetate buffer, pH 5·0 at a flow rate of 30 mL h^{-1}. The protein solution of Fraction 8 was lyophilized. The resulting powder was dissolved in 1·0 mL of buffer and applied to the top of the gel column. The column was eluted with the same buffer at a flow rate of 30 mL h^{-1} at room temperature (20°C) collecting 3·0 mL fractions per tube. Fractions displaying peak proteolytic activity were pooled (15 mL) and designated as curcain (Fraction 9).

Estimation of molecular weight of curcain

The molecular weight of curcain was determined by the gel filtration method of Whitaker (1963) and Andrews (1964) using a Sephadex G-100 column (2·0 × 45 cm). The column was equilibrated with 0·1 M acetate buffer, pH 5·0. The void volume (V_0) was determined using Blue dextran 2000 eluting with the same buffer at a flow rate of 30 mL h^{-1} at room temperature. The column was calibrated with bovine serum albumin, ovalbumin and cytochrome C. 2·5 mg of each protein in 1·0 mL equilibrating buffer was applied to the column and eluted with the same buffer at the same flow rate with that of Blue dextran 2000. Fractions of 3·0 mL/tube were collected and the elution volume (V_e) from the peak mid-point of each of the markers was determined by measuring the optical density of the fractions at 280 nm. Fraction 9 was dialysed against deionized water and lyophilized. The resulting powder was dissolved in the equilibrating buffer in a concentration of 2·5 mg mL^{-1}. One mL of the sample was applied to the column and eluted with the buffer. The elution volume (V_e) of the enzyme protein was determined by measuring the position of protein in the effluent at 280 nm.

Disc electrophoresis of curcain

The polyacrylamide gel electrophoresis of curcain in non-denaturing buffer system was carried out according to the method of Ornstein (1964) and Davis (1964) with slight modification. The standard tube gels of small pore (10·0%, pH 8·8) and large pore (2·5%, pH 6·8) were used in a vertical electrophoresis apparatus. The upper and lower electrode chambers were filled with two times diluted stock buffer (Tris 6·0 g; glycine 28·8 g; water to make 1 L), pH 8·3. Curcain was dissolved in 20% (w/v) sucrose solution containing two drops of 0·5% (w/v) Bromophenol Blue (as marker) in a concentration of 2·0 mg mL^{-1}. Fifty μL of the protein solution (100 μg of protein) was applied to the top of each tube. Electrophoresis was carried out at room temperature by applying a current of 3·0 mA per tube until the dye front reached the bottom.

At the conclusion of the run the gels were released from the tubes and the protein bands were stained with 0·25% (w/v) Coomassie Brilliant Blue in methanol: water: acetic acid (45:45:10) for 1 h, and blanched with 7·5% (v/v) acetic acid in 5·0% (v/v) methanol in water for two days with several changes.

Results and Discussion

A summary of the purification procedure is presented in Table 1. The total protein and the protease activity of different fractions at each purification step was determined. The enzyme extracted with 0–25% ammonium sulphate precipitation (Fraction 4) showed maximum specific activity when azoalbumin was used as substrate. Fraction 4 was dialysed against 0·2 M acetate buffer, pH 5·0 and the enzyme solution (Fraction 7) was utilized for chromatographic separation.

An elution diagram of the enzyme from the CM-cellulose column is given in Fig. 1. The protein pattern in Fig. 1 shows one major peak in fractions 18–27 and one minor peak in fractions 37–41. The fractions under the minor peak pos-

Table 1. Steps in the purification of curcain.

Fraction No.	Purification steps	Volume (mL)	Total protein (mg)	Total activity (units)	Specific activity (units (mg protein)$^{-1}$ ($\times 10^3$)	Yield (%)	Purification
1	Crude extract	400	3200	2·67	0·83	100	—
2	Acetone precipitation	50	640	1·00	1·56	38·0	1·88
3	Mixed-solvent precipitation	50	525	0·83	1·59	31·0	1·92
4	0–25% Ammonium sulphate precipitation	50	450	1·50	3·33	56·2	4·01
5	25–60% Ammonium sulphate precipitation	50	630	0·67	1·06	25·0	1·28
6	60–80% Ammonium sulphate precipitation	20	320	0·02	0·06	0·75	0·07
7	Dialysis of extracted enzyme	50	450	1·58	3·51	59·0	4·23
8	CM-cellulose chromatography	50	8·60	0·137	15·93	5·13	19·20
9	Sephadex G-200 gel-filtration	15	2·85	0·10	35·09	3·75	42·28

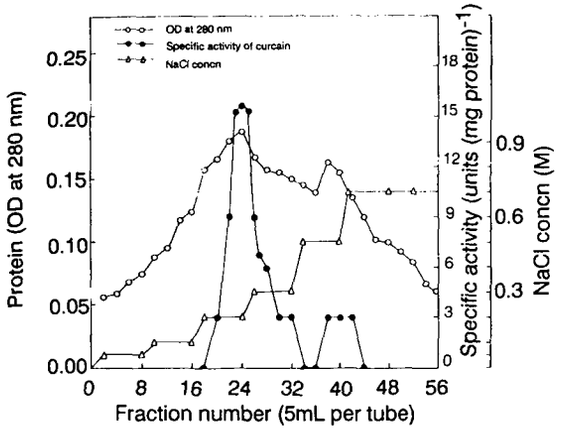

FIG. 1. Purification of curcain on carboxymethyl cellulose.

sesses very little activity compared with the fractions under the major peak, and hence, the enzyme is associated with the fractions under the major peak.

A typical elution pattern of gel filtration with the Sephadex G-200 column is presented in Fig. 2. Azoalbumin was used as substrate to follow the protease activity of the eluted fractions. The protein peaks in the elution diagram reveal that the enzyme sample contains three proteins of different molecular size, but only the protein peak eluted in fractions 26–31 possesses proteolytic activity. The overall purification achieved was 42·28-fold with a yield of about 3·75% (Table 1).

The calibration curve for the standard proteins is shown in

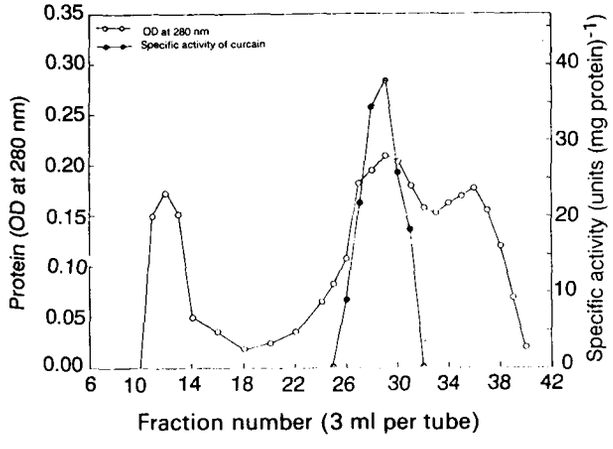

FIG. 2. Purification of curcain by gel-filtration on Sephadex G-200.

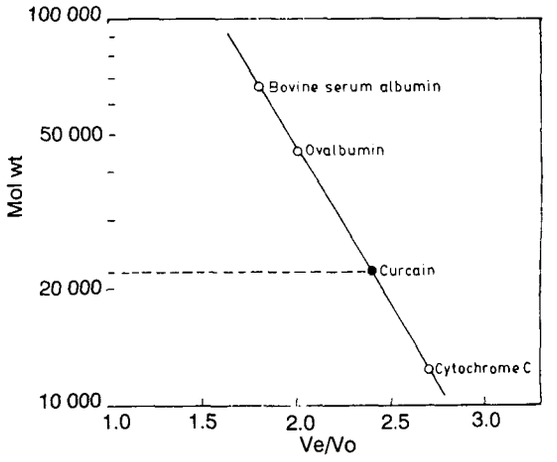

FIG. 3. Calibration curve (semi-log plot) for determination of molecular weight of curcain by gel-filtration with Sephadex G-100.

Fig. 3. By interpolation the molecular weight of curcain was estimated to be 22000 daltons. This molecular weight for curcain is of the same order of magnitude as the molecular weight for a number of other proteolytic enzymes, including pepsin (35000 Bovey & Yanari 1960); ficin (26000 Bernhard & Gutfreund 1956), and carboxypeptidases A and B (34000 Folk et al 1960). Trypsin, α-chymotrypsin and papain also fall into this molecular weight range.

The homogeneity of the protein associated with curcain has been established by carrying out the polyacrylamide gel electrophoresis using a discontinuous buffer system, resulting in a single band of curcain.

It may be concluded that curcain, the protease extracted and purified from the latex of *Jatropha curcas* Linn is a proteolytic agent which might have biochemical and pharmaceutical applications.

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